RNase-neutralized pancreatic microsomal membranes from livestock for *in vitro* co-translational protein translocation

Kurt Vermeire,[1*] Susanne Allan,[2] Becky Provinciael,[1] Enno Hartmann,[2] and Kai-Uwe Kalies[2]

[1]KU Leuven - University of Leuven, Department of Microbiology and Immunology, Virology and Chemotherapy, Rega Institute for Medical Research, B-3000 Leuven, Belgium

[2]Institute of Biology, CSCM, University of Lübeck, Lübeck, Germany

[*]Correspondence: kurt.vermeire@rega.kuleuven.be

Address: Minderbroedersstraat 10, 3000 Leuven, Belgium

Tel: +32 – 16 – 33 73 71

Fax: +32 – 16 – 33 73 40

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ABSTRACT

Here, we demonstrate that pancreatic microsomal membranes from pigs, sheep or cattle destined for human consumption can be used as a valuable and ethically correct alternative to dog microsomes for cell-free protein translocation. By adding adequate ribonuclease (RNase) inhibitors to the membrane fraction, successful *in vitro* co-translational translocation of wild-type and chimaeric pre-prolactin into the lumen of rough microsomes was obtained. Also, the human type I integral membrane proteins CD4 and VCAM-1 were efficiently glycosylated in RNase-treated microsomes. Thus, ribonuclease-neutralized pancreatic membrane fractions from pig, cow or sheep are a cheap, easy accessible and fulfilling alternative to canine microsomes.

Keywords:

cell-free protein translation; pancreatic microsomes; mammalian ER membranes; ribonuclease; cyclotriazadisulfonamide (CADA); translocation inhibitor

All living cells produce proteins that must be translocated across or inserted into hydrophobic biological membranes. In eukaryotic cells, most secretory proteins cross the endoplasmic reticulum (ER) membrane in a co-translational way. Also membrane proteins with hydrophobic transmembrane sequences are integrated into the ER membrane co-translationally [1]. In the ER, proteins are further modified resulting in mature proteins that are signal peptide-cleaved and properly folded with e.g., N-linked glycosylation. To study protein biogenesis *in vitro*, cell free translation mixtures from rabbit reticulocytes are used to translate *in vitro* synthesized mRNA into proteins. However, secretory and membrane proteins rely on ER membranes to get properly translocated and processed during translation [2;3]. Several studies in eukaryotic cells have been performed with membranes from yeast [4-6]. Microsomal membranes isolated from the pancreas of dogs are the main source of microsomes to study cell free *in vitro* protein translocation into mammalian ER [7]. Major drawbacks of the use of dogs are the high cost of obtaining these animals and the ethical question of sacrificing dogs for research purpose.

Membranes from rat liver [8;9] and hen oviduct [10] have been tested by other groups. However, the reproducibility and transport efficiency for analyzed secretory or membrane proteins in these membranes were not always satisfactory. Kaderbhai *et al.* reported the successful usage of rough membranes isolated from the pancreas of sheep sacrificed for research purpose [11], and this system has been exploited by others [12]. Here, we searched for a valuable alternative to canine rough microsomes (RM), and evaluated pancreatic RM from different animals destined for human consumption (Figure 1). In a slaughterhouse, pancreases from sacrificed pigs and cows were isolated, collected and stored on ice. In addition, sheep pancreases were isolated from animals sacrificed under controlled conditions. Next, microsomal membranes from the excised pancreases were isolated based on a protocol described for canine RM [13].

Notably, up to 100,000 equivalents (for definition, see reference [13]) of rough membranes could be isolated from a single sheep pancreas.

To evaluate the biological activity of the isolated microsomes, we examined the translocation of secreted proteins in a cell free *in vitro* translation system. As a reporter protein, we selected the well-studied bovine pre-prolactin (pPL), a secreted protein of 26kDa with an N-terminal cleavable signal peptide of 30 residues [14]. The cloning of bovine pPL in the pGEM4 vector (plasmid pGEMBP1) has been described elsewhere [15]. The DNA was transcribed into mRNA using the T7 RiboMAX^{TM} Large Scale RNA Production System (Promega) and the synthesized mRNA purified with an RNA purification kit (Macherey-Nagel). Next, purified mRNA was used as a template for *in vitro* translation reaction with the rabbit reticulocyte lysate system (Promega), in the presence of [^{35}S]methionine (Perkin Elmer). Translations were performed at 25°C for 45 min. Samples were washed once in a 50 mM HEPES buffer (pH 7.5) containing 80 mM KAc and 2 mM MgAc, and synthesized proteins were isolated by sedimentation at 4°C. Pellets were dissolved in SDS sample buffer for analysis by 4% stacking-12% separating SDS-PAGE and visualized by phosphorimaging (Cyclone Plus; Perkin Elmer). The band intensity of each protein was quantified using the Cyclone Plus accompanying software.

In the absence of RM, pPL was efficiently translated from mRNA (Fig. 2A, lane 1). Translocation of pPL was first evaluated with canine pancreatic microsomal membranes (Promega) that served as a positive control. Addition of rough membranes (RM) from dog to the translation mixture resulted in the generation of signal peptide-cleaved prolactin (Figure 2A; lane 3; solid arrowhead). To verify that cleaved prolactin was translocated into the RM lumen, equal aliquots of the translated material were left untreated or digested with Proteinase K (PK; Roche) on ice for 30 min, in the presence or absence of Triton X-100 (Sigma-Aldrich). Digestion was

terminated by the addition of phenylmethylsulfonyl fluoride (PMSF; Thermo Fisher Scientific). As expected, only signal peptide-cleaved prolactin, which is found inside the microsomes if translocated, was protected from PK digestion (Figure 2A; lanes 3 and 4; solid arrowhead), whereas the cytosolic precursor was degraded in the presence of PK (open arrowhead). Solubilization of the microsomes with the detergent Triton X-100 resulted in the complete digestion of all pPL species (Figure 2A; lane 5). Overall, the translocation efficiency of pPL in dog RM ranged between 19 and 23%.

Our first attempts to translocate proteins in RM from sheep pancreas did not result in any detectable polypeptides (Fig 2A; upper panel, lanes 7 and 8). Also, when we repeated this experiment with RM from porcine or bovine origin, no pPL species could be detected (Fig 2A; lane 7). We hypothesized that the mRNA in the translation mixture was degraded by ribonucleases (RNases) present in the membrane fraction. Therefore, membrane stocks were diluted in membrane buffer (50 mM HEPES/KOH (pH 7.6), 250 mM sucrose and 1 mM DTT) and pretreated with increasing amounts of the recombinant RNase inhibitor RNasin (Promega). For the ovine RM, administration of some RNase inhibitor (7 U/eq) could not yet rescue the RNA in the translation mixture and resulted in a failure of protein translation (Figure 2A; upper panel, lanes 10 and 11). However, when sufficient ribonuclease inhibitors were administered to the ovine RM, nearly half of the pPL species were translocated into the lumen of the microsomes, resulting in a translocation efficiency that was twice as high as compared to the dog RM (Figure 2A; lanes 16 and 17; 48% translocation). Thus, ovine RMs are highly translocation efficient for pPL when the mRNA is rescued in the translation mixture. For the translocation of pPL into porcine RM, only very little RNasin (2.4 U/eq) was needed to prevent mRNA degradation. Maximum pPL translation and translocation were obtained when the RM from pig were neutralized with 6 U RNasin/eq RM (Figure 2A, middle panel, lanes 13 and 14). Overall, with

porcine RM translation efficiencies ranged between 37 and 40%. As shown in Figure 2A, bovine RM were less successful in translating and translocating pPL (translocation efficiency of 31%).

Next, to confirm the biological activity of the different RM, we evaluated the post-translational N-glycosylation of two different type I integral membrane proteins. For this experiment we selected human Cluster of Differentiation 4 (CD4) and human Vascular Cell Adhesion Molecule 1 (VCAM-1) as reporter proteins. Both cell surface membrane proteins are signal peptide-cleaved but also glycosylated when properly translocated into the ER lumen. Translocated CD4 and VCAM-1 will appear as N-glycosylated proteins with an apparent higher molecular weight (i.e., upper protein band on the gel). As shown in Figure 2B, human CD4 was efficiently N-glycosylated in the presence of canine (45%), ovine (46%), porcine (52%) and bovine (41%) RM. Accordingly, human VCAM-1 became N-glycosylated in the presence of the different RMs (Fig. 2B; 65%, 44%, 46% and 28% for canine, ovine, porcine and bovine RM, respectively). Thus, RNase-neutralized microsomes obtained from sheep, pigs and cows performed the requested post-translational modifications with comparable efficiencies as the dog RM.

As we have recently reported the identification of a highly selective human CD4 translocation inhibitor [16], we next assessed if the translocation inhibition by this cylcotriazadisulfonamide (CADA) compound could be observed in membranes from different species. Prolactin containing either the signal sequence of human CD4 ([CD4]-PL) [16] or wild-type preprolactin (WT pPL) was translated and translocated as described for Figure 2A, and was treated with or without 15 µM of CADA. As shown in Figure 2C, the translocation of wild-type preprolactin was unaffected by CADA in the different membranes. However, for [CD4]-PL the translocation of prolactin was clearly inhibited by administration of CADA, as evidenced by the reduced amount of signal peptide-cleaved and PK-resistant processed protein in the CADA-

samples compared to the corresponding control PK-samples (Figure 2C, upper panel). Thus, a comparable inhibitory effect of CADA on [CD4]-PL could be noted in the different membranes: 85.0%, 84.3%, 75.5% and 82.8% translocation inhibition in canine, ovine, porcine and bovine RM, respectively. This is in line with the proposed model of specific binding of the drug to the human CD4 signal peptide, rather than sole interaction with the translocon channel [16].

In conclusion, for investigating protein translocation *in vitro*, rough membrane vesicles of endoplasmic-reticular origin from the pancreas of different livestock animals can be used as a valuable alternative to the dog source. As these animals are destined for human consumption, this alternative source is more convenient and ethically correct. Protein-translocation-competent rough microsomes from sheep pancreas have been used by others [11;12], but the animals were sacrificed for research purpose only. In contrast, regarding the porcine and bovine microsomes, we were able to prepare fully functional rough ER membranes from pancreas that was excised from the animal directly in a slaughterhouse. The use of porcine RMs for *in vitro* protein translocation was reported to be unsuccessful [11], and these microsomes from porcine pancreas required a high salt–wash before being used in ribosome export studies [17;18]. Here, we demonstrated that by mainly neutralizing the RNase activity in the microsomal fraction, rough microsomes from either pig, sheep or cow can be successfully used for studying protein transport and the associated co- or post-translational modifications. By simple pretreatment with RNasin, RMs from slaughtered livestock largely meet or exceed the results obtained with commercially available canine microsomes. In addition, the use of RMs from different species will be very helpful in investigating the universal features of protein translocation across the ER and to unravel the complexity of this protein translation/translocation process. It remains to be analyzed whether these membranes from cattle, sheep and pig are as suitable as membranes from dogs for

specific investigations of protein translocation into the ER, such as cross-linking or the production of reconstituted proteoliposomes.

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Legends to the figures:

Figure 1. Principal layout of the isolation of the pancreas from different animals, extraction of the microsomal membranes and ribonuclease neutralization of the microsomes before usage in cell free *in vitro* translation assays.

Figure 2. Microsomes isolated from livestock are biological active in translocation. A) Full length bovine preprolactin (pPL) was translated in rabbit reticulocyte lysate and translocated into dog and RNasin-treated rough membranes (RMs) from sheep (top panel), pig (middle panel) and cow (bottom panel). Open arrowhead indicates the precursor protein pPL, whereas the signal cleaved (and translocated) mature protein PL is marked by a solid arrowhead. The amount of ribonuclease inhibitor RNasin per equivalent of RM is indicated below each sub-panel. The canine RMs were not treated with RNasin. PK, proteinase K. B) Comparison of the N-glycosylation of type I integral membrane proteins in RMs from different species. The membrane proteins human CD4 and VCAM-1 were translated in rabbit reticulocyte lysate and translocated into canine and RNasin-treated ovine, porcine and bovine RMs. Open arrowhead indicates the precursor protein, whereas the glycosylated species are marked by a star. Bottom panel shows deglycosylation of VCAM-1 by Endo H treatment of porcine RMs. C) Comparable translocation inhibition with CADA in RMs from different species. The secretory CD4-prolactin chimaera protein ([CD4]-PL) and wild-type preprolactin (WT pPL) were translated in rabbit reticulocyte lysate and translocated into canine and RNasin-treated ovine, porcine and bovine RMs in the absence or presence of the selective human CD4 translocation inhibitor CADA (15 µM). Open arrowhead indicates the precursor protein, whereas the signal peptide cleaved (and translocated) mature protein PL is marked by a solid arrowhead. Graph shows the quantification of translocated PL. Similar data were obtained in a repeat experiment with 15 and 150 µM CADA. CTRL, control; CADA, cyclotriazadisulfonamide.

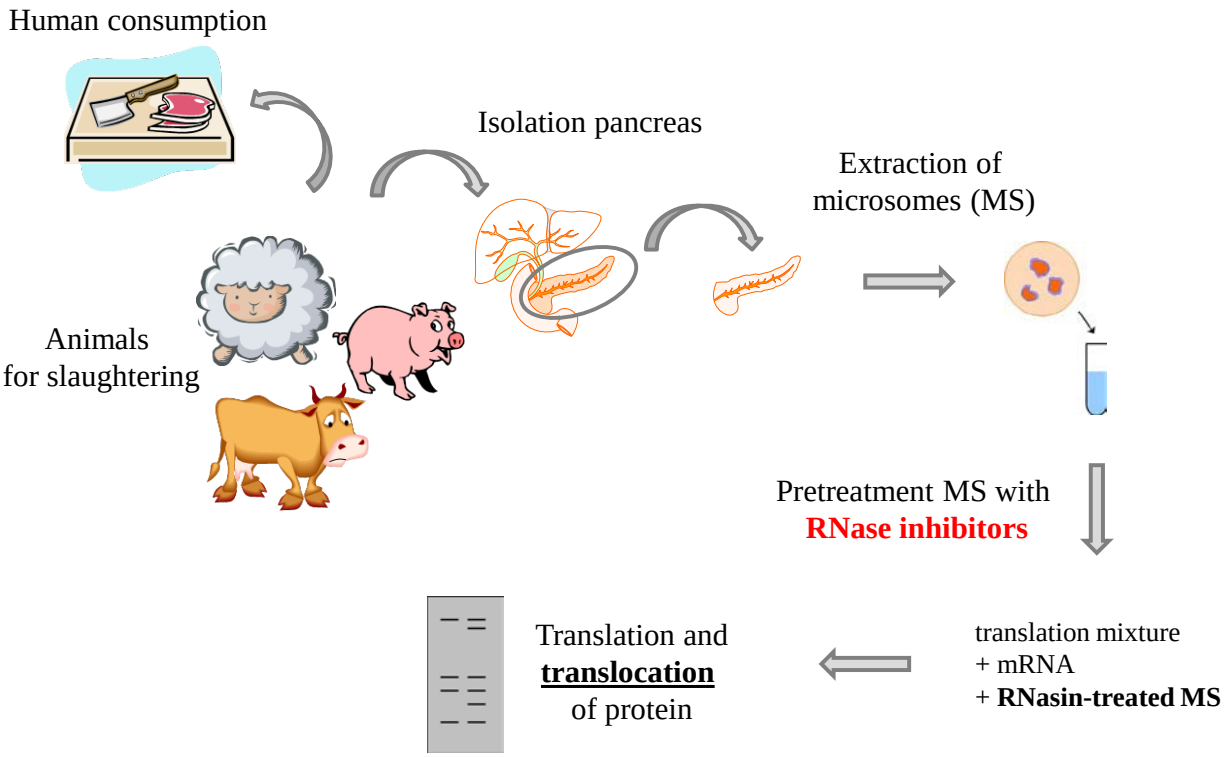
Human consumption
Isolation pancreas
Extraction of
microsomes (MS)
Animals
for slaughtering
Pretreatment MS with
RNase inhibitors
translation mixture
+ mRNA
+ RNasin-treated MS
Translation and
translocation
of protein

FIGURE 1

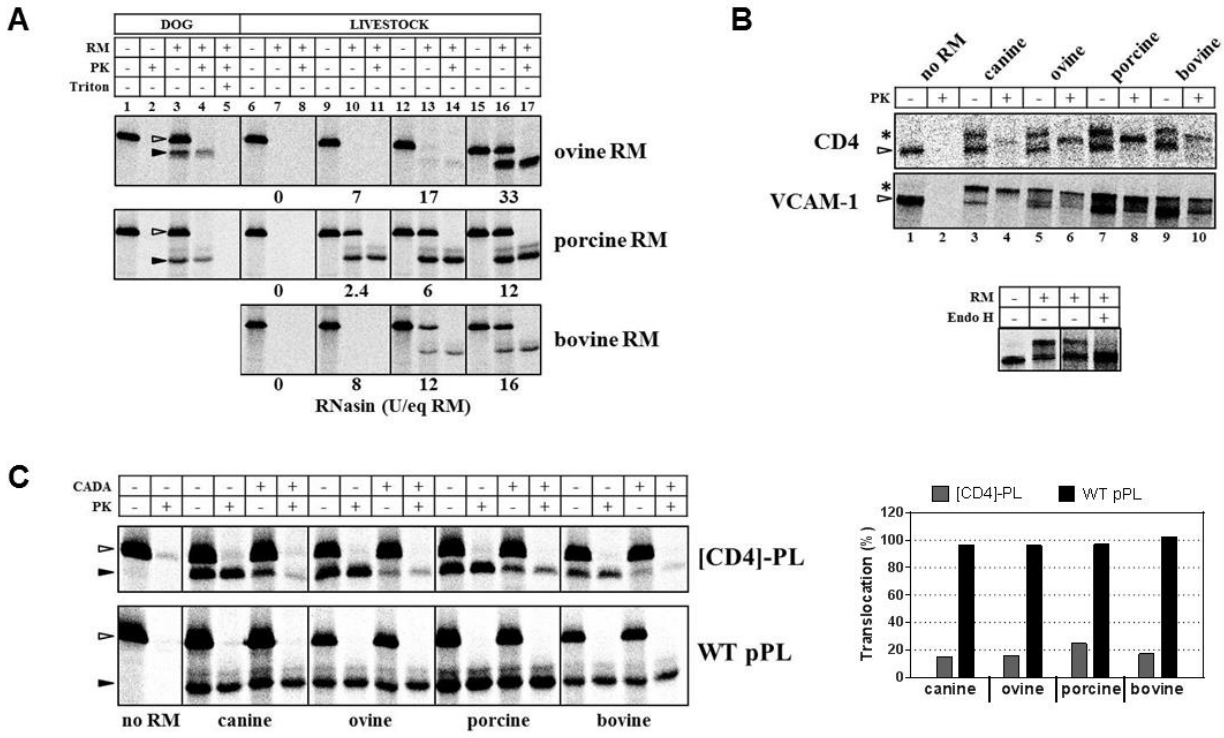
A
DOG
LIVESTOCK
RM
PK
Triton
ovine RM
porcine RM
bovine RM
RNasin (U/eq RM)
B
no RM
canine
ovine
porcine
bovine
PK
CD4
VCAM-1
RM
Endo H
C
CADA
PK
[CD4]-PL
WT pPL
no RM
canine
ovine
porcine
bovine
[CD4]-PL
WT pPL
Translocation (%)
canine
ovine
porcine
bovine

FIGURE 2